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L6: Entry 8 of 8 File: USPT Mar 5, 1996

DOCUMENT-IDENTIFIER: US 5496699 A
TITLE: Detection of allele - specific mutagens

DEPR:
This technique is applicable to the identification in biological fluid of sequences from single copy genes, mutated at a known position on the gene. Samples of biological fluid having soluble DNA (e.g., blood plasma, serum, urine, sputum, cerebral spinal fluid) are collected and treated to deproteinize and extract the DNA. Thereafter, the DNA is denatured. The DNA is then amplified in an allele-specific manner so as to amplify the gene bearing a mutation.

DEPR:
Other techniques may also be used to extract the DNA while preventing the DNases from affecting the available DNA. Because plasma DNA is believed to be in the form of nucleosomes (mainly histones and DNA), plasma DNA could also be isolated using an antibody to histones or other nucleosomal proteins. Another approach could be to pass the plasma (or serum) over a solid support with attached antihistone antibodies which would bind with the nucleosomes. After rinsing the nucleosomes can be eluted from the antibodies as an enriched or purified fraction. Subsequently, DNA can be extracted using the above or other conventional methods.

DEPR:
This method conducts the amplification using four pairs of oligoucleotide primers. A first set of four primers comprises four allele-specific primers which are unique with respect to each other. The four allele-specific primers are each paired with a common distant primer which anneals to the other DNA strand distant from the allele-specific primer. One of the allele-specific primers is complementary to the wild type allele (i.e., is allele-specific to the normal allele) while the others have a mismatch at the 3' terminal nucleotide of the primer. As noted, the four unique primers are individually paired for amplification (e.g., by PCR amplification) with a common distant primer. When the mutated allele is present, the primer pair including the allele-specific primer will amplify efficiently and yield a detectable product. While the mismatched primers may anneal, the strand will not be extended during amplification.

DEPR:
In one embodiment, the length of each allele-specific primer can be different, making it possible to combine multiple allele-specific primers with their common distant primer in the same PCR reaction. The length of the amplified product would be indicative of which allele-specific primer was being utilized with the amplification. The length of the amplified product would indicate which mutation was present in the specimen.

DEPR:
The method of this invention may be embodied in diagnostic kits. Such kits may include reagents for the isolation of DNA as well as sets of primers used in the detection method, and reagents useful in the amplification. Among the reagents useful for the kit is a DNA polymerase used to effect the amplification. A preferred polymerase is Thermus aquaticus DNA polymerase available from Perkin-Elmer as AmpliTaq DNA polymerase. For quantitation of the mutated gene

sequences, the kit can also contain samples of mutated DNA for positive controls as well as tubes for quantitation by competitive PCR having the engineered sequence in known amounts.

DEPR:
A modified competitive PCR could also be developed in which one primer has a modified 5' end which carries a biotin moiety and the other primer has a 5' end with a fluorescent chromophore. The amplified product can then be separated from the reaction mixture by adsorption to avidin or streptavidin attached to a solid support. The amount of product formed in the PCR can be quantitated by measuring the amount of fluorescent primer incorporated into double-stranded DNA by denaturing the immobilized DNA by alkali and thus eluting the fluorescent single stands from the solid support and measuring the fluorescence (Landgraf et al., Anal. Biochem. 182:231-235, 1991).

DEPR:
The deletion mutants with an approximately 20 residue deletion will be derived as previously described (Vallette et al. 1989). In summary, the P1 and P2 primers will be used in an allele-specific manner with the normal DNA or with DNA from the tumor cell line with each specific mutation. Each of these would be paired for amplification with a common primer which contains the sequence of the common primer normally used with either the P1 and P2 allele-specific primers, i.e., "P1-B" or "P2-B" at the 5' end with an attached series of residues representing sequences starting approximately 20 bases downstream, thus spanning the deleted area (common deletion primer 1 and 2, CD1 and CD2). The precise location and therefore sequence of the 3' portion of the primer will be determined after analysis of the sequence of the ras gene in this region with OLIGO (NB1, Plymouth, MN), a computer program which facilitates the selection of optimal primers. The exact length of the resultant amplified product is not critical, so the best possible primer which will produce a deletion of 20-25 residues will be selected. For example, with P2 primers the allele-specific primer for the wild-type will be 5' ACTCTTGCCTACGCCAC 3' complementary to residues 35 to 51 in the coding sequence. To effect a deletion of approximately 20 residues in the complementary strand, the common upstream primer to be used with the wild-type and the three allele-specific primers for mutations in position two of codon 12 will be 40 residues long (CD2) complementary to residues -95 to -78 (the currently preferred common upstream primer for use with P2 allele-specific primers and residues at approximately -58 to -25). The amplified shorter product will be size-separated by gel electrophoresis and purified by Prep-a-Gene (Biorad). DNA concentrations will be determined by the ethidium bromide staining with comparison to dilutions of DNA of known concentration. This approach will be repeated eight times, using the four P1 primers and common primer (CD1) constructed as above, and four times with the four P2 primers and common primer (CD2). These deletion mutants will be amplified, using the same allele-specific primers used to amplify the genomic DNA. Therefore, they can be used subsequently in known serial dilutions in a competitive PCR, as outlined above.

CLPR:
20. A diagnostic kit for detecting the presence of a K-ras mutation in the nucleic acids in biological fluid, wherein the mutation is present in the twelfth codon at position 1, comprising:

CLPR:
21. The diagnostic kit of claim 20 further comprising